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Autoantibodies as diagnostic biomarkers for the detection and subtyping of multiple sclerosis

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ABSTRACT

The goal of this preliminary proof-of-concept study was to use human protein microarrays to identify blood-based autoantibody biomarkers capable of diagnosing multiple sclerosis (MS). Using sera from 112 subjects, including 51 MS subjects, autoantibody biomarkers effectively differentiated MS subjects from age- and gender-matched normal and breast cancer controls with 95.0% and 100% overall accuracy, but not from subjects with Parkinson's disease. Autoantibody biomarkers were also useful in distinguishing subjects with the relapsing-remitting form of MS from those with the secondary progressive subtype. These results demonstrate that autoantibodies can be used as noninvasive blood-based biomarkers for the detection and subtyping of MS.

1. Introduction

Multiple sclerosis (MS) is a neuroinflammatory autoimmune disease that primarily affects white matter of the central nervous system (CNS) (Kis et al., 2008; Reynolds et al., 2011; Sanai et al., 2016). Recent estimates show that between 250,000 and 350,000 people in the United States currently suffer from MS (Sanai et al., 2016; Miller and Hens, 1993). As is the case with many autoimmune conditions, women are disproportionately affected by MS, with a ratio of three women for every one man diagnosed (Sanai et al., 2016; Harbo et al., 2013). The reason for this discrepancy in gender is unknown, but it is speculated to be influenced by hormonal, genetic, or environmental differences (Harbo et al., 2013). Currently, MS is pathologically characterized by subcortical white matter lesions separated temporally and spatially, with microscopic structural defects accruing in the myelin sheaths that insulate axons for proper neuronal firing (Miller and Hens, 1993; Diaz-Sanchez et al., 2006; Bitsch et al., 2000). Demyelination occurs commonly in the white matter of the brain, including in the optic nerve and spinal cord, but later progresses to include gray matter lesions that are readily visualized in magnetic resonance images (MRIs). Common symptoms include lower extremity muscle weakness, paresthesias, vision changes, and later, cognitive decline as the disease progresses (Jurynczyk et al., 2015; Harris and Sadiq, 2014).

Presently, a diagnosis of MS involves a thorough patient history, imaging such as MRI to detect white matter lesions, an electrophysiological examination using evoked potential tests, and cerebrospinal fluid (CSF) analysis to detect the presence of increased immunoglobulin species (Birnbaum, 2006). While some success has been achieved to accurately diagnose and treat the symptoms of some patients, others succumb to progressively worsening disease symptoms and opportunistic conditions (Evlice et al., 2016; Noseworthy, 1994; Daumer et al., 2009). Due to the autoimmune nature of MS, much research attention has focused on specific components of the immune system to attempt to identify and diagnose patients at the earliest possible stage of their disease. For instance, autoantibodies targeting myelin surface proteins, such as myelin oligodendrocyte glycoprotein, myelin basic protein, myelin proteolipid protein, and myelin-associated glycoprotein, have demonstrated either associative or correlative links to MS, however, they currently lack utility as accurate diagnostic biomarkers (Harris and Sadiq, 2014; D'Ambrosio et al., 2015; Schirmer et al., 2014; Axelsson et al., 2011; Greeve et al., 2007; Tomassini et al., 2007). Other autoantibody targets with growing interest are glycans, and include

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anti-GAGA4 or anti-glucose antibodies, as well as other cell surface ion channel proteins like KIR4.1 (Brettschneider et al., 2009; Freedman et al., 2009; Srivastava et al., 2012). Despite the abundance of potential biomarker candidates, thus far there is no definitive biofluid test capable of accurately diagnosing MS or monitoring its progression.

In several previous studies, we demonstrated the utility of blood-borne autoantibodies as sensitive and specific biomarkers capable of diagnosing and staging Alzheimer's and Parkinson's diseases at early stages with high overall accuracy, as well as successfully differentiating them from other neurodegenerative and non-neurodegenerative diseases (E. Nagele et al., 2011; Han et al., 2012; DeMarshall et al., 2016; C.A. DeMarshall et al., 2015). In the present study, our objective was to use this strategy to determine if autoantibodies can also be used as blood-based biomarkers to diagnose individuals with MS using sera from MS subjects afflicted with either the relapsing-remitting MS (RRMS) or secondary progressive MS (SPMS) subtype, the two most prevalent clinical courses of this disease. Roughly 80% of all MS patients are initially diagnosed with RRMS and, during the course of their disease, more than 60% of RRMS patients will transition to SPMS (Compston and Coles, 2002). Our results show that a panel of autoantibody biomarkers can be used to differentiate patients with MS from appropriate age- and gender-matched control subjects with an overall accuracy of 95.0%. We also identified additional autoantibody biomarker panels that are subtype-specific for RRMS or SPMS, and then used each of these panels to successfully differentiate these MS subtypes. Using RRMS-specific autoantibody biomarkers, we were able to differentiate RRMS patients from SPMS patients with 100.0% accuracy. Similarly, SPMS-specific autoantibody biomarkers were capable of differentiating SPMS patients from RRMS patients with 92.0% accuracy. These comparisons demonstrate the potential of autoantibody biomarker panels to effectively and sequentially stage the clinical course of MS, as well as possibly identify the point of transition between subtypes. Finally, MS subjects were also readily distinguished from those with breast cancer, a non-neurodegenerative disease control group, with comparable accuracy.

2. Methods

2.1. Ethics statement

Approval for the use of serum samples in this study was obtained from the Rowan-Stratford Institutional Review Board.

2.2. Study population

Thirty-one relapsing-remitting multiple sclerosis (RRMS) and twenty secondary progressive multiple sclerosis (SPMS) serum samples were obtained from *BioServe Biotechnologies, Ltd.* (Beltsville, MD). Fifteen early-stage PD samples were obtained from the Parkinson's Study Group (Boston, MA), and fifteen stage 3–4 breast cancer samples were obtained from *BioServe Biotechnologies, Ltd*. Healthy control samples were obtained from a variety of sources, including two from *Analytical Biological Systems, Inc.* (Wilmington, DE), twenty-eight from *BioServe Biotechnologies, Ltd.*, and one from *Asterand, Inc.* (Detroit, MI). All samples were handled using standard procedures and stored at − 80 °C until use, and freezer conditions were monitored using Sensaphone 1400 (Phonetics, Inc., Aston, PA). Demographic characteristics of the study population are listed in Table 1.

Table 1
Sample demographics. The number of individuals (n), age, range of age, gender, and ethnicity are listed for each disease and control group.

Group	n	Age		Gender	Ethnicity
		(Years)	(Range)	(% female)	(% Caucasian)
Multiple sclerosis	51	48.8 ± 10.7	25–67	75	96
-Relapsing-remitting	31	45.8 ± 11.1	25–67	81	94
-Secondary progressive	20	53.5 ± 8.0	36–67	65	100
Controls	31	53.7 ± 13.4	30–79	81	100
Early-stage Parkinson's disease	15	63.5 ± 6.8	51–73	80	100
Breast cancer	15	52.3 ± 6.6	45–63	100	87

2.3. Human protein microarrays

To identify autoantibodies in human sera, we used *Invitrogen*'s ProtoArray v5.1 Human Protein Microarrays (Cat. No. PAH0525020, *Invitrogen*, Carlsbad, CA, USA), each containing 9,486 unique human protein antigens (www.invitrogen.com/protoarray). All proteins were expressed as GST fusion proteins in insect cells, purified under native conditions, and spotted in duplicate onto nitrocellulose-coated glass slides. Arrays were probed with serum and scanned according to the manufacturer's instructions using commercially prepared reagents. Microarray slides were blocked (Blocking Buffer, Cat. No. PA055, *Invitrogen*) and then each was incubated with serum diluted to 1:500 in washing buffer. After washing, arrays were probed with anti-human IgG (H + L) conjugated to AlexaFluor 647 (Cat. No. A-21445, Invitrogen). Arrays were then washed, dried, and immediately scanned with a GenePix 4000B Fluorescence Scanner (*Molecular Devices*, Sunnyvale, CA, USA).

2.4. Microarray data analysis

Fluorescence data were acquired by aligning the Genepix Array List onto the microarray image using the Genepix Pro analysis software. The resulting Genepix results files were imported into Invitrogen's *Prospector 5.2* for analysis. The “group characterization” and “two-group comparison” features in the Immune Response Biomarker Profiling (IRBP) toolbox within *Prospector* then enabled M-statistical analysis of the differential autoantibody expression between the two groups being compared. Positive hits were determined by a Z-Factor > 0.4 and a minimum signal intensity of 1500 RFU, which allows for stringent biomarker selection and minimizes the number of false positives. Autoantibodies were first sorted into descending order by difference of prevalence between MS and control groups, and the top 50 most differentially expressed autoantibodies in the MS group were chosen as potential MS diagnostic biomarkers and tested further. Additionally, a second round of biomarker selection was carried out by sorting autoantibodies in descending order by difference of prevalence between control and MS groups. This time, the 50 most differentially expressed autoantibodies in the control group, putatively reflecting the selective depletion of these blood-borne autoantibodies in the MS group, were also chosen as potential diagnostic biomarkers and tested. All data are MIAME compliant and raw data from the microarrays have been deposited in a MIAME compliant database (GEO) under accession number (GSE95718).

Subjects were randomly split into Testing and Training Sets such that both sets included cases and controls matched by age and gender. The Training Set was used to rank candidate protein biomarkers by their predictive power and to establish the diagnostic logic. The initial Training Set for the MS group consisted of 26 MS and 16 control samples; the remaining samples were relegated to the independent Testing Set, containing 25 MS and 15 control subjects. The predictive classification accuracy of the selected biomarkers in the Training Set, Testing Set, and in both sets combined was tested with *R*'s *Random Forest* (*RF*; v 4.6–10), using the default settings (O'Bryant et al., 2014; Breiman, 2001). Selected biomarkers were tested with the *RF* model algorithm, and classification accuracy is reported in a confusion matrix and misclassifications as an out-of-bag (OOB) error score. Receiver

Operating Characteristic (ROC) curves, widely used to evaluate the utility of diagnostic tests, were generated using *R* (3.02) packages ROCR (v 1.0-5) and pROC (v 1.7.3) (O'Bryant et al., 2011). Based on the determined optimal number of autoantibody biomarker panel members, a final model was constructed using these biomarkers and their associated Training Set logic and further tested with the independent Testing Set subject samples.

Using the same Training and Testing Set strategy outlined above, we performed an additional round of biomarker discovery using only *RF*, instead of prevalence difference, to select potential biomarkers. After M-statistical analysis by *Prospector*, the data were analyzed using the "variable importance" function in *RF*, which is the prediction accuracy of the OOB error score reported for each decision tree, and also for each individual permutated biomarker. The difference between the two values was averaged over all trees and normalized by the standard error. The 50 most differentially depleted biomarkers in the MS group compared to controls based on the normalized variable importance score were chosen as potential diagnostic biomarkers and further analyzed for their diagnostic value as reported below.

3. Results

3.1. Selection of a panel of autoantibody biomarkers for diagnosis of MS

We first sought to identify a panel of autoantibodies capable of detecting the presence of MS pathology in a mixed-subtype population of MS patients. Serum samples from 51 MS patients with a clinical diagnosis of either relapsing-remitting MS (RRMS) or secondary progressive MS (SPMS), the two most prevalent MS subtypes, and thirty-one age- and gender-matched control samples were randomly separated into either a Training Set (16 RRMS, 10 SPMS and 16 controls) or Testing Set (15 RRMS, 10 SPMS and 15 controls), each containing roughly equal proportions of both MS subtypes. Training and Testing Set sera were used to probe commercially available human protein microarrays containing 9486 protein targets. Autoantibody profiles from the MS and control subjects in the Training Set were compared using *Prospector* microarray analysis software, which then identified 54 autoantibodies with significantly ($P < 0.05$) higher prevalence in the MS group compared to the control group in the Training Set as potential diagnostic biomarkers. From this list, the top 50 most differentially expressed autoantibodies in the MS group were chosen as a working diagnostic biomarker panel.

3.2. Verification of differentially expressed MS biomarkers via Training and Testing Set analysis

The 50 most differentially expressed autoantibody biomarkers chosen from the MS Training Set were evaluated for their prediction accuracy using *Random Forest* (*RF*). Using the 50 selected biomarkers in *RF*, MS subjects were distinguished from age- and gender-matched control subjects in the Training Set (n = 42; 26 MS, 16 controls) with an average 72.4% prediction accuracy based on five replicate runs. We next evaluated the classification potential of the 50 biomarkers along with the *RF* Training Set logic to classify MS in the Testing Set subjects, an independent group of samples that played no role in biomarker selection. *RF* correctly classified an average of 82.5% of MS and controls among Testing Set subjects (n = 40; 25 MS, 15 controls), again using an average of five replicate runs.

3.3. Selection and verification of useful MS biomarkers that are selectively depleted from the blood

Biomarker selection described above was based on the increased production and expression of autoantibody biomarkers in the blood, presumably in response to MS-associated cell and tissue debris production. With MS known to have a strong autoimmune component, we next examined the possibility that disease onset and progression may instead be linked to a selective depletion of autoantibodies that are normally present in the blood. To test this, we used the autoantibody profile expression data generated by *Prospector* from the Training Set samples described above. A total of 3076 autoantibodies with a significantly ($P < 0.05$) lower prevalence in the MS group compared to the control group were identified. From this list, the 50 most differentially depleted autoantibodies in the MS group were chosen as the new working diagnostic biomarker panel. The new panel of depleted MS autoantibody biomarkers chosen from the Training Set were re-verified as significant predictors using *RF*. MS subjects in the Training Set (n = 42; 26 MS, 16 controls) were distinguished from matched controls with an average 95.8% prediction accuracy. Furthermore, *RF* correctly classified 95.0% of MS and controls among Testing Set subjects (n = 40; 25 MS, 15 controls). Finally, combining both Training and Testing Set samples and using the Training Set logic, *RF* successfully distinguished MS from controls with an overall accuracy of 96.8%. Results for all Training and Testing Set comparisons mentioned above are presented as an average of five replicate runs. ROC curve analysis of the overall utility of the depleted MS biomarkers for detection of MS in the Testing Set subjects revealed an area under the curve (AUC) of 0.94, indicating excellent classification accuracy (Fig. 1). Diagnostic sensitivity, specificity, and positive- and negative-predictive values (PPV and NPV) for the 50 depleted MS biomarkers used to evaluate the Testing Set subjects are shown in Tables 2 and 3. Subsequent tests and comparisons carried out in the present study described below refer to the use of the depleted autoantibody biomarker panel described here and shown in Supplementary Table 1, unless otherwise noted.

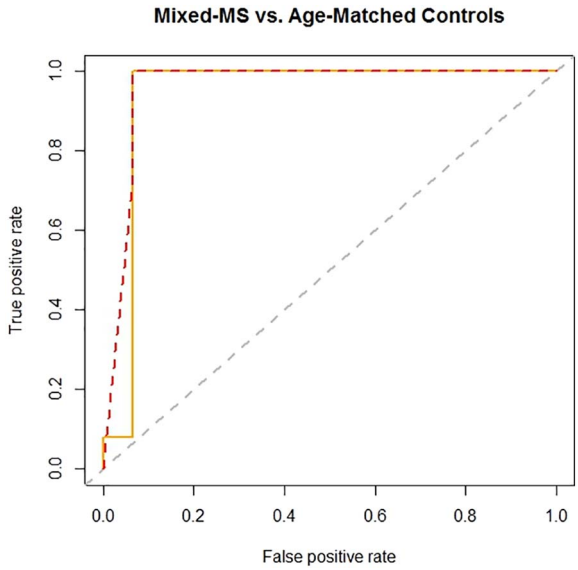

Fig. 1. Biomarker analysis and Receiver Operating Characteristic (ROC) curve assessment of the utility of autoantibody biomarkers for the detection of MS. ROC assessment of autoantibody biomarkers for detection of mixed-MS in Testing Set subjects. Comparison of MS (n = 25) vs. age- and gender-matched controls (n = 15) using a panel of 50 (orange line) or a panel of 3 (red line) MS-specific biomarkers demonstrates that these biomarker panels can be used to detect MS with high overall accuracy. The dashed line represents the line of no discrimination. The ROC AUC, sensitivity, and specificity values for the panels of 50 and 3 biomarkers are shown in Table 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Exchanging Training and Testing Sets yields similar biomarker panels with comparable diagnostic accuracy

To further demonstrate and confirm the utility of depleted autoantibodies as biomarkers for the detection of MS pathology in a separate set of patient samples, we carried out a second round of MS

Table 2
Diagnostic results using a panel of 50 and a panel of 3 biomarkers to mixed (both subtypes included) MS. Diagnostic performance was assessed using *RF*. Using Testing Set samples, *RF* successfully distinguished mixed subtype MS subjects (n = 25) from age-matched and gender-matched controls as well as those with breast cancer with high overall accuracies. *RF* was unable to accurately distinguish MS subjects from those with early-stage PD using the selected biomarkers.

	50 markers			3 markers		
MS (n = 25) vs.	Age matched controls	Early-stage PD	Breast cancer	Age matched controls	Early-stage PD	Breast cancer
n	15	15	15	15	15	15
Sensitivity	100.0	48.0	100.0	100.0	76.0	100.0
Specificity	86.7	26.7	100.0	86.7	6.7	100.0
PPV	92.6	52.2	100.0	92.6	57.6	100.0
NPV	100.0	23.5	100.0	100.0	14.3	100.0
Overall accuracy %	95.0	40.0	100.0	95.0	50.0	100.0
Overall error %	5.0	60.0	0.0	5.0	50.0	0.0

biomarker discovery. In this round, we exchanged the subjects used in the Training and Testing Sets and compared the identities and diagnostic performance of the second round of depleted MS biomarkers with those chosen in the first round. There was a 60% overlap between the second round biomarkers and those chosen in the first round. Using this new panel of 50 second round biomarkers, *RF* was able to correctly classify over 99.0% of MS and controls in Testing Set subjects (sensitivity = 100.0%; specificity = 93.8%; PPV = 96.3%; NPV = 100.0%; ROC AUC = 1).

3.5. Validation of the MS biomarker panel using two biomarker selection strategies: RF vs. prevalence difference

We next carried out an additional and unbiased MS biomarker selection process where *RF* was allowed to independently choose 50 potentially useful depleted MS biomarkers, instead of ranking biomarkers based on prevalence difference through *Prospector* as described above. The panel of 50 *RF*-selected biomarkers (Supplementary Table 2) was able to correctly classify MS and controls in Testing Set subjects with 92.5% overall accuracy based on an average of five replicate runs, thus with comparable accuracy to both panels derived from prevalence difference described above. Importantly, 38 of 50 (76%) of the *RF*-selected biomarkers overlapped with the differentially depleted biomarkers that were selected based on prevalence difference as described above.

3.6. Fewer than 50 depleted autoantibody biomarkers are sufficient for accurate detection of MS

To determine the minimum number of autoantibody biomarkers required to achieve the best diagnostic accuracy, the 50 depleted MS biomarkers were first sorted from the highest to lowest relative importance. Then, biomarkers were successively removed one by one from the bottom of the list until the overall diagnostic accuracy based on the biomarkers remaining began to decline significantly. Using this approach, we determined that a panel of three biomarkers (the top three biomarkers presented in Supplementary Table 1) was the minimum number required to maintain an effective diagnostic accuracy in Testing Set subjects, demonstrating an overall accuracy of 92.5% (sensitivity = 96.0%; specificity = 86.7%; ROC AUC = 0.95) for distinguishing MS subjects from age- and gender-matched controls (Fig. 1; Tables 2, 3).

3.7. Disease specificity of the selected depleted biomarkers for MS

We next evaluated the disease specificity of the original selected panel of depleted biomarkers described above and in Supplementary Table 1 for the detection of MS, with the goal of determining whether these biomarkers could successfully differentiate MS subjects from those with other neurological and non-neurological diseases. To eliminate the possibility that the MS biomarkers were simply detecting nonspecific disease, the same 25 MS sera from Testing Set subjects were compared to sera obtained from 15 subjects with stage 3–4 breast cancer and 15 subjects with early-stage PD. Using the original panel of 50 depleted biomarkers, MS sera were readily distinguished from breast cancer sera with an overall accuracy of 100.0% (sensitivity = 100.0%; specificity = 100.0%; ROC AUC = 1) (Tables 2, 3). By contrast, MS subjects could not be accurately distinguished from early-stage PD subjects, with results showing 40.0% overall accuracy (sensitivity = 48.0%; specificity = 26.7.3%; ROC AUC = 0.72). The inability to distinguish MS from early-stage PD could indicate a substantial overlap in disease pathology, leading to the production of comparable patterns of disease-associated biomarkers.

3.8. Subtyping of MS: discrete autoantibody biomarker panels can distinguish relapsing-remitting MS from secondary progressive MS

We next asked if autoantibody biomarkers could be used to distinguish between different subtypes of MS. Currently, while the majority of MS patients are initially diagnosed as RRMS, a significant fraction of these patients will eventually progress to SPMS for unknown reasons. To address this aspect of MS disease transition and progression, we performed two additional rounds of biomarker discovery to generate panels of subtype-specific expression biomarkers. RRMS (n = 31) and SPMS (n = 20) samples were separated into a Training (n = 26; 16 RRMS, 10 SPMS) and Testing Set (n = 25; 15 RRMS, 10 SPMS), using the same strategy as described above. The top 50 most differentially expressed autoantibody biomarkers in each MS subtype compared to the other were selected and verified as significant using the methods described above. Using RRMS-specific biomarkers and the *RF* logic derived from the Training Set, RRMS sera were readily distinguished from SPMS sera with an overall accuracy of 100% in both Training and Testing Set comparisons. Similarly, using SPMS-specific biomarkers and *RF* logic derived from the Training Set, SPMS sera were readily distinguished from RRMS sera with an overall accuracy of 96.2% in the Training Set, and 92.0% in the Testing Set.

Table 3
ROC curve assessment of the diagnostic utility of the top 50 and top 3 depleted, mixed subtype MS biomarkers. ROC curve analyses (Testing Set subjects only) showing the diagnostic utility of the top 50 and top 3 depleted biomarkers for distinguishing MS subjects from age-matched controls and from early-stage PD and breast cancer. Area under the curve (AUC) values at 95% confidence are listed along with values for sensitivity and specificity derived from ROC curve output data.

MS (n = 25) vs.	50 markers			3 markers		
	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Age matched controls (n = 15)	0.94 (0.82, 1)	1 (1, 1)	0.93 (0.80, 1)	0.96 (0.87, 1)	0.96 (0.88, 1)	0.93 (0.80, 1)
Early-stage PD (n = 15)	0.72 (0.56, 0.88)	0.80 (0.64, 0.96)	0.60 (0.33, 0.87)	0.61 (0.44, 0.78)	0.48 (0.32, 0.68)	0.87 (0.67, 1)
Breast cancer (n = 15)	1	1	1	1	1	1

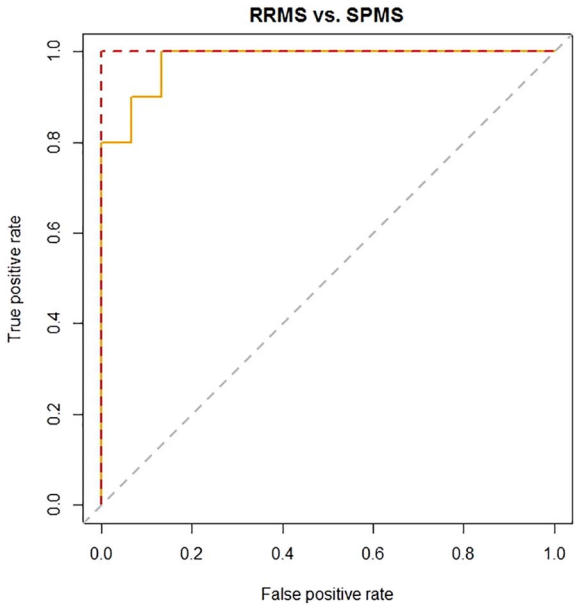

Fig. 2. Biomarker analysis and Receiver Operating Characteristic (ROC) curve assessment of the utility of autoantibody biomarkers for the subtyping and pathological progression of MS. Comparison of Testing Set relapsing-remitting MS (RRMS) subjects (n = 15) vs. Testing Set secondary progressive MS subjects (n = 10) using a panel of 50 RRMS (red line) or SPMS (orange line) specific biomarkers demonstrates that these biomarkers can be used to accurately distinguish between these two different stages of MS progression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Results from ROC curve analyses of these comparisons are presented in Fig. 2, and the identities of the biomarkers in the RRMS-specific and SPMS-specific panels are presented in Supplementary Tables 3 and 4, respectively. These results confirm that, although RRMS and SPMS are different stages of the same disease and are expected to share biomarkers, the panels of subtype-specific autoantibody biomarkers selected here along with their corresponding diagnostic logic were capable of differentiating the pathologies associated with each subtype of MS progression.

4. Discussion

Previously published work by our group has demonstrated the utility and potential efficacy of using human protein microarrays as an accurate and reliable platform for the discovery of blood-based autoantibodies that serve as powerful diagnostic indicators or biomarkers of ongoing disease (E. Nagele et al., 2011; Han et al., 2012; DeMarshall et al., 2016; C.A. DeMarshall et al., 2015). The strength and versatility of this novel approach to MS and other disease diagnostics lies in the proposed role of autoantibodies in the maintenance of body-wide homeostasis through clearance of tissue debris produced in response to ongoing pathology (Nagele et al., 2013). Our biomarker discovery strategy has focused on identifying differentially expressed autoantibodies in disease groups, such as early- and mild- moderate stages of Alzheimer's disease (AD) and Parkinson's disease (PD), relative to healthy control subjects. Using this approach we were not only able to diagnose and stage early phases of both of these diseases, but also to differentiate them from other closely related neurological diseases with high overall accuracy.

In this study, our objective was to determine if panels of blood-based autoantibody biomarkers can be identified with the potential to diagnose MS and distinguish different clinical subtypes of the disease. Sera from a total of 112 subjects were analyzed, including 51 mixed-MS patients diagnosed with either RRMS or SPMS, the two most prevalent MS subtypes that together constitute the vast majority of all diagnosed cases. Using a panel of 50 differentially depleted autoantibody biomarkers, we demonstrate here that it is possible to distinguish MS subjects from healthy age- and gender-matched control subjects with high overall accuracy, sensitivity, and specificity. Additionally, the selected biomarker panel was also capable of successfully differentiating MS subjects from those with stage 3–4 breast cancer, a non-neurodegenerative control group. Lastly, subtype-specific autoantibody biomarker panels and their corresponding diagnostic logic were capable of successfully differentiating between RRMS and SPMS, two clinically relevant MS subtypes representing two discrete phases of the same disease.

The autoantibody biomarker panel used here to distinguish MS patients from matched controls (see Supplementary Table 1) targets a wide variety of constituent proteins, but this list does not include the more well-known proteins that have already been linked to this disease. Identified biomarkers include dehydrogenases, regulatory proteins, voltage-gated potassium channel subunits, kinases, and transcription factors, among others. Based on available literature and database searches, some proposed functions of these selected biomarkers include neuronal differentiation, nerve signal regulation, innate immunity processes, cell motility, RNA modification, transcription/translation regulation, and glycolipid biosynthesis. Although much research effort in the field of MS has previously concentrated on attempts to utilize autoantibodies to specific myelin components as biomarkers of the disease, results thus far have been disappointing and the focus is now shifting towards including a host of antibodies to other proteins as potential biomarkers as described in a recent review by D'Ambrosio et al. (2015). As we continue to learn more about the triggers and mediators of MS pathology and, in turn, MS subtype-specific pathology, we will undoubtedly need to look “outside of the box” containing suspected favorites to discover novel biomarkers which may be the key to elucidating the underlying mechanisms of the disease.

As mentioned above, in previous studies detailing the discovery and testing of blood-based autoantibody biomarkers with utility for diagnosing and staging of AD and PD, the most useful biomarkers selected were autoantibodies exhibiting a higher titer in diseased subjects than in controls (DeMarshall et al., 2016; C.A. DeMarshall et al., 2015; Han et al., 2012; E. Nagele et al., 2011). This pattern agrees with the concept that these autoantibodies are produced in response to debris emanating from regions of pathology, implying a function for autoantibodies in the clearance of this debris from the blood and tissues (C. DeMarshall et al., 2015). It also suggests that such biomarkers would be useful for disease monitoring in patients under treatment by their physicians or in clinical trials, where a beneficial effect would coincide with reduction in debris production from the region of pathology as well as a corresponding reduction in the titers of autoantibodies charged with their clearance. By contrast, in the present study on MS, autoantibodies with lower or depleted titers were found to be the most sensitive, accurate and thus useful biomarkers for disease diagnosis. This suggests that the binding of certain autoantibodies to available targets in regions associated with MS pathology is driving their selective depletion, and could also be playing a causal role in MS. In line with this concept, we have previously provided strong evidence that brain-reactive autoantibodies are ubiquitous in human blood and that, under conditions of blood-brain barrier compromise, they gain access to the brain interstitium and can bind to exposed targets on the surfaces of neurons and glia (Levin et al., 2010; R.G. Nagele et al., 2011; Nagele et al., 2013). Indeed, in the brains of patients with Alzheimer's disease, the same neurons showing particular vulnerability to AD pathological changes, including intraneuronal beta-amyloid deposition, are also the cells that are the most immunoglobulin G (IgG)-positive. This raises the possibility that chronic IgG binding to neuronal surfaces under conditions of blood-brain barrier breach may play a role in AD pathological changes and facilitate amyloid deposition in the affected brain (R.G. Nagele et al., 2011). Here, we propose that this same mechanism is also operating in MS patients, where transient or chronic access of autoantibodies to their targets on the myelin sheath or axonal membranes at the nodes of

Ranvier contribute to a dampening or blocking of nerve impulse transmission at the affected region. Of course, causal factors precipitating the intervals of access of autoantibodies to neuronal targets are unresolved, but are likely to involve transient increases in blood-brain barrier permeability. Further work is required to determine if this is the case.

In addition to autoantibodies being pursued as potential biomarkers for the diagnosis and progression of MS, recent studies have highlighted many other promising candidates that include a wide range of biofluid and imaging biomarkers. For example, a study by Villoslada et al. identified specific patterns of serum metabolites, including hormones, lipids, and amino acids associated with MS and the severity of disease expression (Villoslada et al., 2017). Additionally, Lim et al. examined tryptophan metabolism and were able to use serum kynurenine pathway signatures to successfully differentiate between clinical MS subtypes with an accuracy of 83% in Testing Set subjects (Lim et al., 2017). Recent work by Håkansson et al. evaluated a variety of known neurodegenerative and neuroinflammatory biomarkers in CSF for their prognostic value in patients with clinically isolated syndrome and RRMS. Results revealed that higher baseline levels of neurofilament light chain predicted disease activity in patients with 85% accuracy over the course of a 2 year follow-up period (Hakansson et al., 2017). Lastly, Shepard et al. described a novel mapping technique to measure quantitative increases in thalamic T_2 values in RRMS subjects that was shown to have utility in distinguishing them from healthy control subjects with high accuracy (ROC AUC = 0.913) (Shepherd et al., 2017).

This study has a number of strengths and weaknesses. First, as major strengths, it describes a potential diagnostic approach to MS that employs only a small volume of blood, is relatively non-invasive, and independent of diagnostic imaging such as MRI. Another strength is the ability to develop and use separate panels of autoantibody biomarkers to distinguish between RRMS and SPMS, two clinically distinct subtypes with distinctive pathological disease courses. This finding supports our hypothesis that autoantibody profiles can change with the progression of disease, with each disease and disease stage having its own unique autoantibody signature or profile. We propose that autoantibody profiles truly are responsive to and reflect disease-associated cell and tissue debris production in regions of evolving pathology or the initial binding of brain-reactive autoantibodies to newly available targets as a result of blood-brain barrier compromise. If true, then this information may be useful for determining which profile changes might indicate whether or not a patient will progress or has progressed from one stage to the next, such as in the transition from RRMS to SPMS.

This study also has some weaknesses. First, it is a small proof of concept study intended to address the question of whether our diagnostic strategy with autoantibodies as biomarkers and human protein microarrays as a detection platform can be used to detect MS and differentiate between different clinical MS subtypes. Accordingly, we acknowledge that additional studies using larger cohorts will certainly be needed to confirm the encouraging results presented here. Another weakness is the inability of the chosen biomarker panel, at least using this small sample cohort, to distinguish MS subjects with high accuracy from those with early-stage PD, a closely related neurodegenerative disorder. This could potentially be due to an overlap in pathology-specific biomarkers. Fine-tuning the selection of biomarkers that are better able to delineate these two diseases will no doubt require a larger cohort of MS and early-stage PD subjects. However, as a practical note, even if unresolved, the strikingly different symptom profiles between MS and PD patients make it unlikely that one disease would be mistaken for the other in the clinical setting. In addition, our previous study has shown that PD biomarkers can readily distinguish PD from MS (C.A. DeMarshall et al., 2015) Finally, due primarily to difficulties in procuring sufficient numbers of samples, we were unable at this time to include or test the accuracy of our MS biomarker panels on primary progressive or progressive-relapsing MS patient samples in this study.

In conclusion, we report data representing a “proof of concept” study for a sensitive and specific blood test for the general diagnosis of MS and two of its subtypes. MS represents a wide range of symptoms, clinical presentations, and natural histories, and therefore encompasses a truly heterogeneous patient population – conditions that can often complicate a straightforward diagnosis. Monitoring the course and progression of the disease is dependent on early and accurate diagnoses, which allow earlier treatment intervention. While patients will undoubtedly benefit from earlier, accurate diagnosis and monitoring throughout the course of their disease, the lack of a well-developed, readily accessible and affordable diagnostic tool has hindered progress in the fields of MS research and treatment. For instance, the availability of a diagnostic blood test would be useful in differentiating between a patient experiencing a clinically isolated syndrome triggered by MS and one that is due to other causes. Such a diagnostic would also be expected to have utility as a screening tool that would allow physicians to appropriately direct their patients to seek additional confirmatory tests for MS. In the long term, our diagnostic strategy may also be applicable to verify early enrollment of patients into clinical trials, as well as serve as a means to monitor a patient's response to a particular treatment regimen through documentation of the subsequent loss of MS-relevant biomarkers. It could also be used as a prognostic indicator of impending relapses or worsening conditions/disease progression, as well as the transition between clinical subtypes. Lastly, we have previously demonstrated the utility of using autoantibodies as sensitive and specific biomarkers of early-stage AD and PD. Now, the addition of MS to this list further validates the potential of our strategy as a multi-disease diagnostic approach. We believe that this approach can be applied to most known diseases, regardless of type or tissue of origin; however, the strategy for biomarker selection may need to be tailored to best reflect the disease stage and progression of pathology specific to each disease.

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Competing interests

The authors have the following competing interests: R. Nagele has received research funding from the Michael J. Fox Foundation, the Osteopathic Heritage Foundation, GlaxoSmithKline, the Foundation Venture Capital Group, the Boye Foundation and the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. R. Nagele is also Co-Founder of Durin Technologies, Inc., serves as its Chief Scientific Officer and has received consulting fees. He may accrue revenue in the future based on patents submitted by Rowan University wherein he is a co-inventor. B. Belinka is also Co-Founder of Durin Technologies, Inc., serves as its Chief Executive Officer and has received consulting fees. He may accrue revenue in the future based on patents submitted by Rowan University. A patent has been submitted for the MS autoantibody biomarker panel. There are no marketed products to declare.

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